Lead-Induced Inclusion Bodies: Composition and Probable Role in Lead Metabolism

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Lead-induced inclusion bodies in renal tubular cells of rats have been studied *in vitro* after isolation by differential centrifugation. The inclusion bodies are insoluble in physiological media but may be dissolved in denaturants like $6M$ urea and sodium deoxycholate. They contain about 40–50 μg of lead/mg protein, but only about 10% of this is tightly bound. They also contain calcium, iron, zinc, copper, and cadmium. The protein is rich in glutamic and aspartic acids, glycine and cystine. When dissolved in $6M$ urea, the protein migrates as a single band on acrylamide gel electrophoresis and has a molecular weight of 27,500. It is suggested that the inclusion bodies function as an intracellular depot of nondiffusible lead.

Further studies have been directed toward finding a free, unaggregated lead-containing protein fraction. Nuclear proteins from kidneys of lead-toxic rats were separated into NaCl-, Tris-, and NaOH-soluble fractions and an insoluble acidic fraction. A quantitatively small lead-containing protein was found in the $0.14M$ NaCl fraction. Amino acid composition, electrophoretic mobility, molecular weight, and ability to bind lead are similar to those of insoluble inclusion body protein. The possible role of this soluble lead-binding protein in the formation of nuclear inclusion bodies is at present time not certain. These studies do suggest, however, that protein-bound lead in renal tubular cells may be partitioned between insoluble and nondiffusible morphologically discrete inclusion bodies and a soluble, extractable fraction which is presumably diffusable.

The pathological or adverse health effects of a potentially toxic metal are related not only to the total content of metal in a particular organ system but also, and perhaps more importantly, to the manner in which the metal is partitioned between extra and intracellular sites. In kinetic models of the flow of metals between body compartments or among cell components, it is generally postulated that the metal must exist in two forms, a non-diffusible form which is usually metabolically inactive, and a diffusible, metabolically active form that is associated with microligands and transportable across cell membranes. Other papers in this conference are concerned with the partitioning of lead between red blood cells and circulating blood plasma. This level of lead partitioning is particularly significant with regard to the interpretation of blood lead levels and the equilibrium that must exist among blood lead, lead in the extracellular space, and tissue lead levels.

Similar questions exist regarding the intracellular partitioning of lead in cells of the body, particularly in parenchymal cells of organs which must be engaged in transcellular transport of lead such as kidney and liver.

In this paper the composition and possible role of lead-protein complexes in the intracellular metabolism of lead will be discussed, with particular emphasis on the nature of

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the protein forming lead-induced nuclear inclusion bodies.

Methods

For all the experiments, white Sprague-Dawley male rats weighing 150–200 g were used. Diet consisted of pulverized laboratory chow; powdered lead acetate equivalent to 1% lead was mixed with the diet. Control rats were fed pulverized diet without added lead. All animals were offered tap water *ad libitum*. Animals were killed by decapitation, and kidneys were immediately removed.

Electron Microscopy

Samples of renal cortex and pellets of inclusion bodies were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide; thin sections were stained with uranyl acetate and examined with a JEOLCO-100B electron microscope.

Isolation of Inclusion Bodies

The inclusion bodies were isolated by differential centrifugation in sucrose gradient and sonication to disrupt nucleoli as previously described (*1*). Purity of the inclusion bodies in the isolation procedure was followed by light and electron microscopy.

Chemical Analyses

Metals were measured by atomic absorption spectroscopy by use of a Perkin-Elmer Model 306 equipped with a Delves cup and deuterium background corrector. Protein was measured by the method of Lowry (*2*).

Amino acid composition of isolated inclusion bodies was determined by automated ion exchange column chromatography following acid and basic hydrolysis. For acid hydrolysis, a 2 mg portion of isolated inclusion bodies was added to 2 ml of 6N HCl (redistilled) in an evacuated tube, frozen and warmed twice, and then sealed and placed in an oven at 106 ±2°C for 22 1/2 or 70 hr.

Solubility Characteristics

Preparations of isolated inclusion bodies were incubated with aqueous media (NaCl, NaOH, HCl), organic solvents, chelating agents, urea, and sodium dodecyl sulfate as previously described (*3*).

Gel Electrophoresis

Disc gel electrophoresis of protein fractions was performed by using 7% polyacrylamide gels, a current of 5 mA, and Trisglycine buffer, pH 8.3. Molecular weight estimates were obtained by use of sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis according to the method of Weber and Osborn (4).

Fractionation of Nuclear Proteins from Kidneys of Lead-Poisoned Rats

Nuclear proteins were extracted into five classes by the commonly used method of Steele and Busch (5). The lead content of each fraction was determined (Table 1).

Table 1. Fractionation of nuclear proteins.*

Extraction media	Lead content, μg/mg protein
0.14M Nacl	1.6±1.3
0.01M Tris	1.7±1.5
2.00M NaCl	1.5±1.0
0.05N NaOH	19.2±2.3
Residual	19.5±6.2

* By the method of Steele and Busch (5). Rats fed 0.75% Pb in water for 16–20 weeks.

The four soluble fractions were then electrophoresed on polyacrylamide gel with the use of Tris–glycine buffer pH 8.3, and the major protein bands were eluted from the gel and measured for lead. No lead-containing protein migrated into the gel when the alkali-soluble acidic nuclear protein fraction was electrophoresed, reflecting the fact that the lead is bound to very high molecular weight proteins.

Results

Electron Microscopy

Ultrastructural changes were present in the proximal tubular lining cells as pre-

viously described (*3*). Figure 1 shows a typical lead-induced intranuclear inclusion body, independent of the nucleolus. A second smaller inclusion body is located in the cytoplasm in an indented part of the nuclear membrane.

Metals Analysis

Table 2 summarizes the analysis for a number of metals in the isolated inclusion bodies. They do contain much more lead than any other metal tested, but other metals are contained in the inclusion bodies in measurable amounts, particularly calcium, with lesser amounts of iron, zinc, copper, and cadmium; measurement of mercury was not attempted.

Table 2. Metals associated with inclusion bodies.

	Metal, μg/mg protein
Lead	55.8
Calcium	13.4
Iron	5.0
Zinc	1.3
Copper	0.3
Cadmium	0.05

Amino Acid Composition of Inclusion Body Protein

Amino acid composition of the hydrolyzed inclusion body protein shows that the protein is very acidic, rich in glutamic and aspartic acids, glycine, and cystine (Table 3). Whether the inclusion bodies contain a single pure protein or a mixture of similar
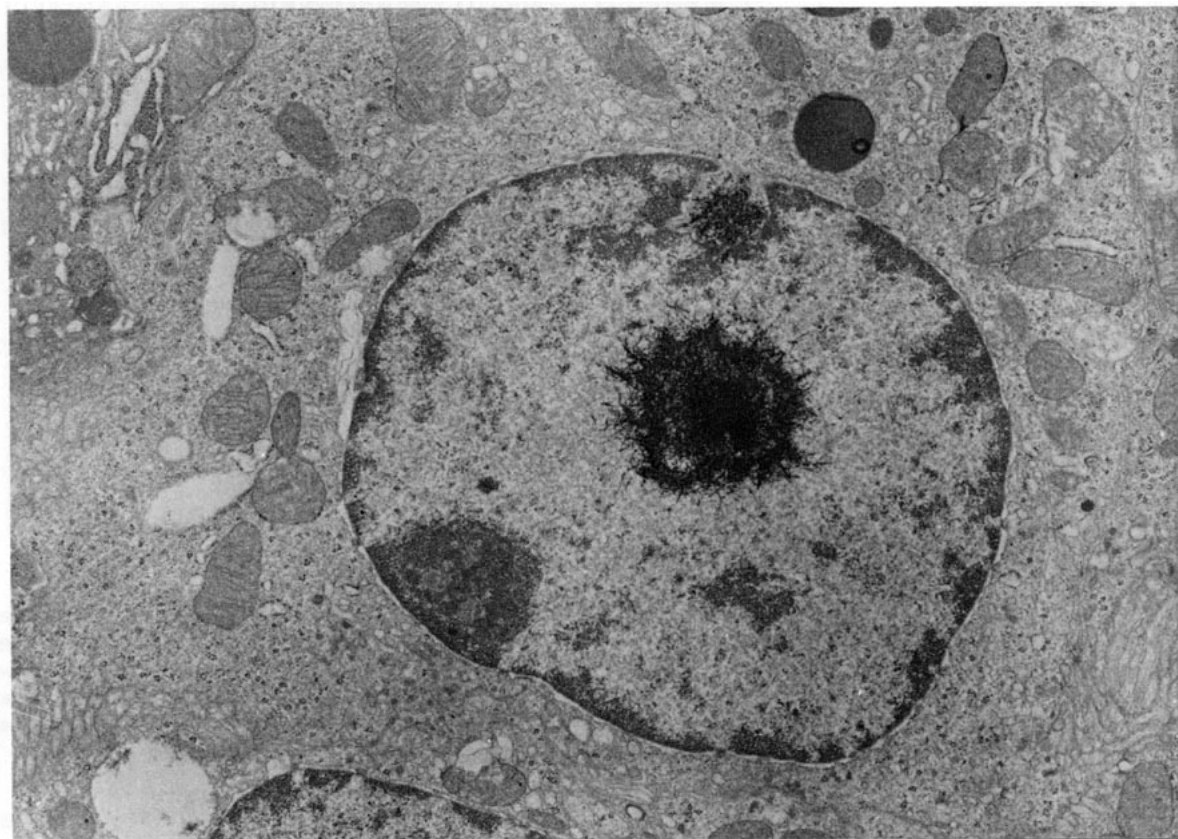

FIGURE 1. Proximal renal tubular lining cell from a lead poisoned rat containing a nuclear inclusion body characterized by dense central core and fibrillary margin. The nuclear inclusion body is distinct from the nucleolus. A small inclusion body is located within invaginated nuclear membrane. ×1200.

Table 3. Amino acid content of lead inclusion protein.

Amino acid	Concn, mole-%
asp	9.7
thr	7.9
ser	6.4
glu	9.9
pro	4.4
tyr	1.2
gly	12.6
ala	8.3
val	4.7
ile	3.2
leu	7.2
try	3.4
½–cys	4.0
met	1.2
lys	7.3
arg	4.9
hist	1.3
ϕ–ala	2.2

$$\frac{Asp + Glu}{Lys + His + Arg} = 1.44 \text{(inclusions)}.$$

Residual Acidic Fraction = 1.3–1.5.

acidic proteins is uncertain. The amino acid composition and solubility characteristics of inclusion bodies are similar to those of the residual or insoluble acidic fraction of nuclear proteins found in normal cell nuclei (*6, 7*).

Electrophoresis of Urea-Solubilized Inclusion Body Protein

Figure 2 shows a polyacrylamide gel electrophoresis of inclusion body protein dissolved in 6M urea. The protein migrates rapidly as a single band to the distal end of the gel consistent with the behavior of a strongly acidic protein. A sample containing 0.1 mg of protein was applied to the column; the protein had 57.6 μg lead/mg protein. Most of the lead remained on top of the gel. Only 3.1 μg lead/mg of protein migrated with the protein band. This indicates that only about 10% of the lead in the inclusion body remains bound to the migrating band. Molecular weight of this protein has been determined by SDS–polyacrylamide gel electrophoresis using marker proteins, and is approximately 27,500.
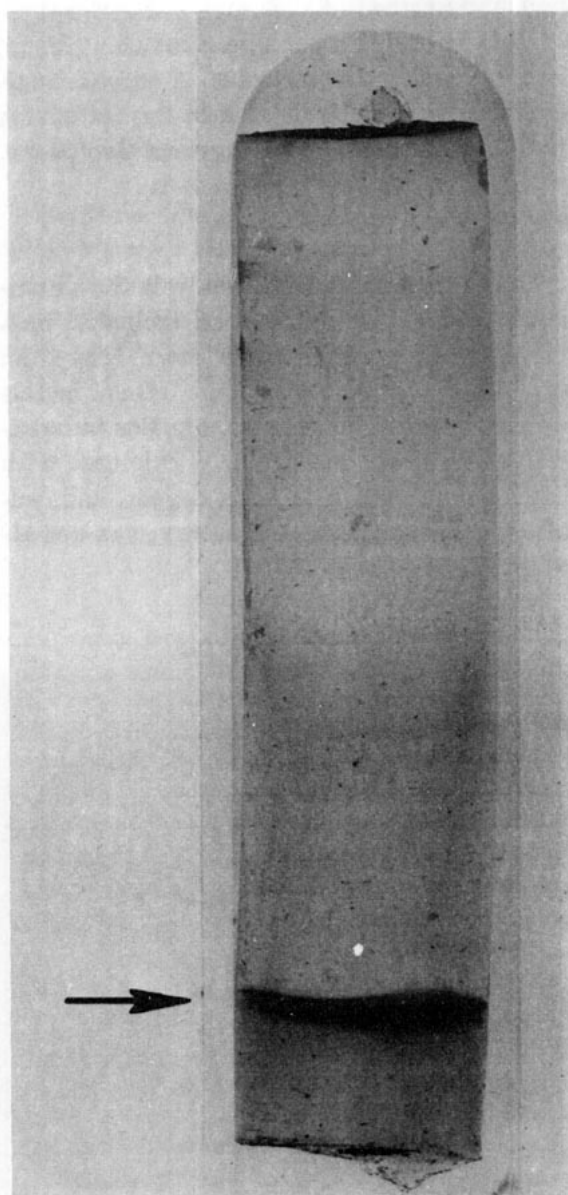

FIGURE 2. Polyacrylamide gel (7%) with a single protein band obtained after electrophoresis of the soluble portion of nuclear inclusion bodies incubated in 6M urea, pH 9, for 24 hr at 37°C. The electrophoresis was run by use of a Tris–glycine buffer (pH 8.3) that also contained 6M urea.

Electrophoresis of 0.14M Sodium Chloride Extractable Fraction of Nuclear Proteins

Figure 3 shows gel electrophoresis of the total 0.14M sodium chloride extractable fraction. Electrophoresis of other nuclear
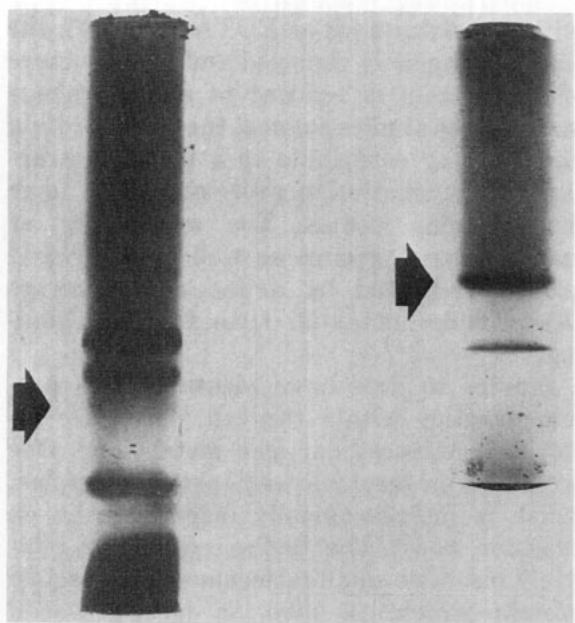
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FIGURE 3. Polyacrylamide gels (7%) showing the electrophoretic pattern of the 0.14M NaCl-extractable nuclear protein fraction (left) and the protein band obtained after concentration of the protein that binds large amounts of lead (right). The arrows point to the lead containing protein bands. The electrophoresis was run by use of a Tris-glycine buffer (pH 8.3).

of protein migration is from top to bottom. This protein has been eluted from several gels like the one on the left and again electrophoresed under the same conditions. The result is the gel on the right. Metal content, amino acid composition, electrophoretic mobility and molecular weight of this band have been determined as they were for the inclusion body protein. The results of these analyses and a comparison with inclusion body protein is shown in Table 4.

The sodium chloride extractable nuclear protein may be saturated *in vivo* with up to 50 μg lead/mg protein. It also contains zinc, copper, and cadmium but no calcium or iron. Both this protein and inclusion body protein migrate as a single band on electrophoresis. They cannot be compared directly since inclusion body protein must be electrophoresed with urea in the media, but electrophoresis with sodium dodecyl sulfate shows that both proteins have a similar molecular weight. Their amino acid compositions are very similar. The only apparent differences are that the inclusion bodies are insoluble while the other protein is soluble and that the two proteins are extractable from different nuclear protein fractions.

protein fractions did not identify a lead containing protein band. The 0.14M sodium chloride fraction contains several bands, but the only one that contains lead is a light band indicated by the arrow. The direction

Discussion

Lead-induced inclusion bodies have been shown by a number of techniques to be composed of a lead–protein complex (*1, 8–*

Table 4. Comparison of the soluble lead-binding protein with protein from inclusion bodies.

	Inclusion protein	Soluble protein
Metal binding	Pb up to 70 μg/mg protein	Pb up to 50 μg/mg protein
	Also Zn, Cu, and Cd	Also Zn, Cu, and Cd
	Ca present	No Ca
	Fe present	No Fe
Electrophoresis (pH 8.3)	Single band	Single band with similar mobility
Molecular weight	27,500	28,500
Amino acid composition	Gly, glu, asp >10%	Same
	cys, 2.6%; trp, 5.0%	cys, 3.2%, trp, 4.8%
	No met	No met
	Highly acidic protein	Same
Differences	Insoluble	Soluble
	In residual fraction of nuclear protein	In 0.14M NaCl fraction of nuclear protein

10). About 90% of the lead in the kidney of lead-toxic rats is found in these lead–protein complexes (11). This has suggested to us that the inclusion bodies represent a nondiffusible depot of lead in the cell. Accumulation of lead in this manner results in a relatively small increment of lead in other portions of the cell such as the endoplasmic reticulum and mitochondria. The formation of these bodies, therefore, is important in the cells of those organs which must metabolize or transport lead (12). Inclusion bodies are common in hepatic parenchymal cells (12, 13) as well as renal tubular cells following excessive exposure to lead and they have also been induced experimentally in osteoclasts of bone (14) and glial cells of the brain near the surface of the cerebral cortex (12). Their formation seems to be a universal phenomena in most living things. They have been found in many species of mammals (11, 13–16), in birds (17, 18), and in moss leaf cells (19).

Although the inclusion bodies are almost always found in nuclei, they are sometimes found in the cytoplasm (16). Whether inclusions are formed only in the cytoplasm and then move across the nuclear membrane or whether they may form in either site is not known. However, they are almost always found in the nucleus. We have tried to detect evidence of movement of the protein fibrils through nuclear pores and as yet have no positive evidence that this can occur.

One of the serious difficulties in studying the protein in these bodies is that the inclusions are virtually insoluble. They are insoluble in all physiologic media. Treatment with lipid solvents such as chloroform, butanol, ether, or acetone mixtures alters their morphology somewhat but does not remove the lead. Electron microscopy of isolated inclusion bodies incubated with organic solvents shows some dissolution of matrix and loosening of fibrils but the lead–protein content is not altered. It is suggested that the inclusions contain a small amount of lipid but that the lipid is not essential to the metal binding (3).

When the inclusion bodies are incubated with a chelator such as EDTA or ADPC, the lead is completely removed and the structure of the protein is reduced to an amorphous mass. These studies suggest that lead–protein binding may contribute to a tertiary structure or aggregation of protein units to form the inclusion bodies. The availability of many carboxyl groups as well as sulfhydryl groups, indicated by amino acid analyses (3) provides potential sites for lead binding.

Studies to date have identified two protein moieties within the cell which are involved in intracellular lead metabolism. One protein is an insoluble lead–protein complex, which is morphologically discernible as an inclusion body. The bodies contain a relatively insoluble small molecular weight acidic protein which, in vivo, is loosely bound with a major fraction of the intracellular lead. It also contains lesser amounts of other metals. This protein moiety provides a site for the deposition of nondiffusible lead in the cells, but because of its extreme insolubility it is doubtful that this protein, at least in this form, is involved in the intracellular transport of lead.

The second protein is soluble and extractable with isotonic sodium chloride. Amino acid composition, electrophoretic mobility, molecular weight, and ability to bind lead are similar to those of insoluble inclusion body protein. The possible role of this soluble lead-binding protein in the formation of nuclear inclusion bodies is at the present time uncertain. These studies do suggest, however, that protein-bound lead in renal tubular cells may be partitioned between insoluble, nondiffusible morphologically discrete inclusion bodies and a soluble, extractable protein which is presumably diffusible and involved in the transport of lead.

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